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10 March 1988 (10.03.88)

(54) Title: SERUM FREE TISSUE CULTURE MEDIUM CONTAINING POLYMERIC CELL-PROTECTIVE AGENT

(57) Abstract

An essentially serum-free tissue culture medium characterised in that it includes in solution a polymer which acts as cell protective agent by reducing film drainage around cells. Preferred polymers are polyethylene glycol, polyvinyl pyrollidone and polymers containing one or more alkylene oxide. This tissue culture medium is of particular use in the cultivation of cells requiring attachment under aeration. Methods of cultivation of such cells are also claimed.

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AMENDED CLAIMS
[received by the International Bureau on 29 January 1988 (29.01.88).
original claims 1-24 replaced by new claims 1-21 (3 pages)]

1. A method of culturing mammalian cells which require attachment comprising the following steps:
   1. Culturing the mammalian cells in a serum containing medium in a reaction vessel provided with a support medium to which the cells will attach until the cells have attached thereto and grown to the desired cell concentration;
   2. Decanting the serum containing medium;
   3. Replacing the decanted medium with an essentially serum-free medium which includes in solution a non-toxic polymer which is capable of acting as a cell protective agent by reducing film drainage around the cells and the support medium; and
   4. Aerating the culture.

2. A method as claimed in claim 1 in which the non-toxic polymer present in the serum-free medium is selected from the group comprising polyethylene glycol and polyvinyl pyrollidone.

3. A method as claimed in claim 1 in which the non-toxic polymer present in the serum-free medium is a polymer which contains one or more alkylene oxides.

4. A method as claimed in claim 3 in which the alkylene oxide contains 2 or 3 carbon atoms.

5. A method as claimed in claim 3 or claim 4 in which the polymer is a copolymer of propylene oxide and ethylene oxide.

6. A method as claimed in any one of claims 3 to 5 in which the polymer of alkylene oxides is present in the medium in a concentration of 0.1 to 1.0% by weight.

7. A method as claimed in claim 1 in which the reaction vessel is divided into a culture region and a cell-free region, the culture region being separated from the cell-free region by means of a filter through which

culture medium but not cells may pass.

8. A method as claimed in Claim 7 in which the aeration of the culture is conducted by bubbling a gas into the cell-free region of the reaction vessel.

9. A method as claimed in Claim 8 in which the aeration of the culture is achieved by aerating essentially serum-free medium free of cells in an aeration chamber remote from the reaction vessel, the aerated medium then being transferred into the reaction vessel.

10. A method as claimed in claim 9 in which the essentially serum-free medium free of cells which is subjected to aeration is, at least in part, medium taken from the cell-free region of the reaction tank.

11. A method as claimed in any one of claims 1 to 7 in which the aeration is carried out by means of a gas lift present within the reaction vessel.

12. A method as claimed in any one of claims 1 to 11 in which the mammalian cell is genetically engineered to contain DNA encoding a polypeptide, such that growth of the cells results in production of the polypeptide.

13. A method as claimed in claim 12 in which the polypeptide is recovered and purified from culture medium taken from the cell-free region of the reaction vessel.

14. A method as claimed in claim 12 or claim 13 in which the DNA coding for the polypeptide is under the control of an inducible promoter, the promoter being induced by a component of the essentially serum-free medium.

15. A method as claimed in any one of claims 12 to 14 in which the mammalian cells are a continuous cell line.

16. A method as claimed in claim 15 in which the continuous cell line is Chinese hamster ovary cell line.

17. A method as claimed in any one of claims 12 to 16 in which the polypeptide is human growth hormone.

18. A method as claimed in any one of claims 1 to 17 in which the essentially serum-free medium is also

essentially protein-free.

19. A method as claimed in any one of claims 1 to 18 in which the culture is agitated in a manner such that shear forces generated are low.

20. A method as claimed in any one of claims 12 to 19 in which the polypeptide is recovered from culture medium continuously removed from the reaction vessel, and fresh essentially serum-free medium is continuously added to the reaction vessel.

21. A method of culturing a mammalian cell which require attachment substantially as hereinbefore described with reference to Example 1 or 2.

22. A method of culturing mammalian cells which require attachment comprising the following steps:

1. culturing the mammalian cellsin a serum containing medium in a reaction vessel provided with a support medium to which the cells will attach until the cells have attached thereto and grown to the desired cell concentration;
2. decanting the serum containing medium;
3. replacing the decanted medium with an essentially serum-free medium; and
4. aerating the culture in a manner such that the mammalian cells are not directly exposed to the aeration.

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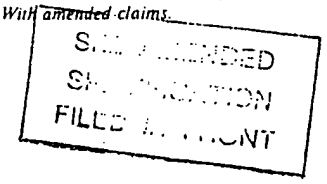

(54) Title: SERUM FREE TISSUE CULTURE MEDIUM CONTAINING POLYMERIC CELL-PROTECTIVE AGENT

(57) Abstract

An essentially serum-free tissue culture medium characterised in that it includes in solution a polymer which acts as cell protective agent by reducing film drainage around cells. Preferred polymers are polyethylene glycol, polyvinyl pyrollidone and polymers containing one or more alkylene oxide. This tissue culture medium is of particular use in the cultivation of cells requiring attachment under aeration. Methods of cultivation of such cells are also claimed.

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SN	Senegal
SU	Soviet Union
TD	Chad
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US	United States of America

SERUM FREE TISSUE CULTURE MEDIUM CONTAINING POLYMERIC CELL-PROTECTIVE AGENT

The present invention relates to an improved culture medium for use in culturing mammalian cells requiring attachment, under aeration.

The present invention further relates to a method for producing polypeptides from mammalian cell lines into which DNA coding for the desired polypeptide has been inserted.

Developments in recombinant DNA technology have made it possible to insert DNA coding for a desired polypeptide into the genome of continuous mammalian cell lines. These cell lines have certain advantages over the use of prokaryotic hosts such as E. coli. These advantages include the ability to secrete and glycosylate proteins, and the ability to process genetic material in an identical fashion to that occurring in cells in the body. To be weighed against these advantages is the fact that the use of recombinant mammalian cells for the production of proteins involves large scale tissue culture, mostly in antibiotic free medium. Tissue culture requires more precise environmental control and more rigorous design and operation of equipment to avoid contamination than does prokaryotic cell culture. A further disadvantage in the use of mammalian cells is that the medium used in the cultivation of these cells typically contains serum. The presence of serum greatly adds to the complexity of isolation and purification of the desired polypeptide product from the culture media.

BACKGROUND ART

It is possible to place cells used in tissue culture in two broad categories: (1) Those which grow free in suspension culture, such as hybridoma cells, and (2) those which grow attached to surfaces. The former cells can be grown in suspension culture providing consideration is taken of the fact that the cells do not possess cell walls and are hence sensitive to shear forces. Cells in the

second category require increased surface area of cell growth in order to scale-up production. Various devices have been described in the literature to provide this increased surface area, including devices providing glass honeycomb surfaces, surfaces wrapped as sheets and glass spheres of several mm diameter. A full review of the various approaches described in the literature appears in the paper by Glacken et al (1983) (1).

Another approach is to use smaller diameter spherical particles which can be constructed from a range of materials including cross linked dextran, polystyrene or glass. The advantage of these particles, sometimes referred to as microcarriers, is that it is possible to achieve very high surface areas per unit mass of beads (5000 cm^2/gm) with particles with diameters of the order of 100-200 um. An extension of this approach is the use of porous microcarriers which allow the cells to grow inside the beads as well as on the outer surface. Another possibility is to use even smaller diameter latex beads (of the order of 1 um diameter) to act as nucleation sites for the attachment of cells to these beads and to each other. Under certain conditions it may be possible to induce the cells to attach to each other to form agglomerates of cells or flocs. Once cells are attached to either microcarriers or beads or flocs, it is possible to grow the cells in suspension culture. Equipment for growing cells in suspension culture is in routine use and the use of this equipment avoids the problem of developing technology using different equipment such as membrane devices. One of the difficulties encountered, however, with cells grown on microcarriers is that at the cell densities achieved oxygen limitation may occur. While such a limitation may be overcome using known aeration techniques such aeration may cause the cells to be disassociated from the support medium, when the culture

medium is serum-free.

DISCLOSURE OF THE INVENTION

The present inventors have developed a novel tissue culture medium, which enables the aeration of cultures of cells attached to a support medium, without the consequent detachment of the cells in the absence of serum. In addition, the present inventors have developed novel methods for the cultivation of cells requiring attachment.

In a first aspect the present invention consists in an essentially serum-free tissue culture medium characterized in that the culture medium includes in solution a polymer which acts as a cell protective agent by reducing film drainage around the cells.

Without wishing to be bound by scientific theory it is believed that the polymer which acts as a cell protective agent prevents the disassociation of the cells from the support medium and lysis of the cells, when the cells are exposed to direct aeration in the absence of serum, by forming a protective film around the cell/support agglomerate. It is believed that this protective film around the cell/support agglomerate prevents the film of culture medium which surrounds the cells from draining away when the cell/support agglomerate comes into contact with air bubbles. This draining of culture medium is referred to as film drainage. It is also believed that this film may provide a barrier which protects the cells from physical damage caused by the air bubbles.

It is preferred that the polymer is non-ionic and low foaming.

In a preferred embodiment of the present invention the polymer is either polyethylene glycol, polyvinyl pyrollidone or a polymer containing one or more alkylene oxides. It is preferred that the alkylene oxides contain two or three carbon atoms. It is particularly preferred

that the polymer is a copolymer of propylene oxide and ethylene oxide.

In a further preferred embodiment of the present invention the polymer of alkylene oxides is present in a concentration of 0.1 to 1.0%.

In another preferred embodiment of the present invention the tissue culture medium is essentially protein-free.

In a second aspect, the present invention consists in a method of culturing a mammalian cell which require attachment comprising the following steps:

1. Culturing the mammalian cells in a serum containing medium in a reaction vessel provided with a support medium to which the cells will attach until the cells have attached thereto;
2. Decanting the serum containing medium;
3. Replacing the decanted medium with an essentially serum-free medium; and
4. Aerating the culture in a manner such that the mammalian cells are not directly exposed to the aeration, or directly aerating the culture containing the mammalian cells wherein when the aeration is direct the essentially serum-free medium includes in solution a polymer which acts as a cell protective agent by reducing film drainage around the cells.

In a preferred embodiment of this aspect of the present invention the reaction vessel is divided into a culture region and a cell-free region by means of a filter through which culture medium but not cells can pass.

Indirect aeration of the culture medium is carried out by bubbling gas into the essentially serum-free culture medium in an aeration chamber remote from the reaction vessel. The aerated medium is then transferred back into the reaction vessel. Alternatively, gas is bubbled into a cell-free region of the reaction vessel.

When the serum-free culture medium includes the polymer which acts as a cell protective agent by reducing film drainage around the cells and the aeration is direct, it is preferred that aeration is conducted using an air lift positioned within the reaction vessel.

In preferred embodiments of this aspect of the present invention the mammalian cells are a continuous cell line, particularly Chinese hamster ovary cell line, into which DNA coding for a desired polypeptide, particularly human growth hormone (hGH), has been inserted.

Preferably the DNA coding the desired polypeptide is under the control of an inducible promoter, which is induced by a component of the essentially serum-free medium. Further, in a preferred embodiment the essentially serum-free medium is essentially protein-free.

The agitation of the culture may be achieved using an impellor such as an anchor type impellor at a rate at which shear forces generated are low. Obviously other impellor configurations can be used providing the shear forces generated are low.

In a further preferred embodiment the essentially serum-free medium is added continuously and the desired polypeptide recovered continuously.

In order that the nature of the invention may be better understood preferred forms thereof are hereinafter described by way of example, with reference to the accompanying drawings, in which:-

Figures 1, 2 and 3 are representations of reactor systems used in preferred embodiments of the invention illustrating various means of aerating the cultures.

Figs. 1 and 2 show indirect aeration of the culture medium whilst Fig. 3 shows direct aeration using an air lift positioned within the reaction vessel.

Example 1

A Chinese hamster ovary (CHO) cell line was

transfected with DNA coding for human growth hormone linked to a strong regulatable promoter/enhancer sequence. Further details of the genetic construction are given in WO 86/04920.

Cells were stored in liquid nitrogen ampoules, then reconstituted by growing in either Roux flasks or roller bottles. Cells were harvested using EDTA, washed in PBS and contacted with pretreated microcarriers. The microcarriers were either dextran based (Cytodex 1 and 2, Pharmacia or polystyrene based, Biosilan, Nunc). Seeding densities of 6-10 cells per carrier were found suitable and resulted in the cells attaching and growing with minimum lag and doubling times of approximately 18 hrs. The medium was a synthetic medium containing 5 or 10% FCS. To build up the number of cells needed to inoculate larger scale vessels, it was possible to add fresh microcarriers to populations of confluent microcarriers and colonisation of the fresh carriers would occur. The desired inoculum of microcarriers was transferred to the configuration of the fermentor shown in Fig. 1.

Cells were grown in a serum containing medium until the microcarriers were confluent. The growth media was then removed, the beads being retained in the reactor by the 100 micron stainless steel screen shown in Fig. 1. This screen, designated 10, divides the reaction vessel into a culture region 11 and cell-free region 12. The cells and beads were washed in 1 volume of phosphate buffered saline (PBS) and induction medium for hGH production added. This essentially serum-free medium consisted of:

APPENDIX 1

INORGANIC SALTS	mg/l
$CaCl_2$ (anhyd.)	162.15
$CuSO_4 . 5H_2O$	0.0013
$Fe(NO_3)_3 . 9H_2O$	0.05

$FeSO_4 . 7H_2O$	0.417
KCl	352.5
KH_2PO_4	30.62
$MgCl_2$ (anhyd.)	23.33
$MgSO_4$ (anhyd.)	61.48
NaCl	6958.5
$NaHCO_3$	1338
$NaH_2PO_4 (H_2O)$	62.5
$ZnSO_4 . 7H_2O$	0.432
$Na_2HPO_4 . 7H_2O$	125
AMINO ACIDS	
L-Alanine	9.0
L-Asparagine.H_2O	15.0
L-Arginine.HCl	253.0
L-Aspartic acid	13.0
L-Cysteine HCl.H_2O	35.13
L-Cystine.2HCl	31.29
L-Glutamic acid	15.00
L-Glutamine	438.0
Glycine	23.0
L-histidine HCl.H_2O	42.0
L-Isoleucine	56.4
L-Leucine	65.6
L-Lysine HCl	109.5
L-Methionine	19.5
L-Phenylalanine	38.0
L-Proline	35.0
L-Serine	31.5
L-Threonine	59.4
L-Tryptophan	10.0
L-Tyrosine (disodium salt)	59.83
L-Valine	58.7
VITAMINS	
Ascorbic acid	7.5
Biotin	0.0037

D-Ca pantothenate	2.119
Choline chloride	8.98
Folic acid	2.66
i-Inositol	12.61
Nicotinamide	2.02
Pyridoxal HCl	2.03
Riboflavin	0.22
Thiamine HCl	2.169
Vitamin B_{12}	0.68
OTHER COMPONENTS	
D-Glucose	3151
HEPES	3570
Sodium pyruvate	110
Hypoxanthine (disodium salt)	2.7
Linoleic acid	0.045
Putrecine 2HCl	0.081
Thioctic acid (Lipoic)	0.103
Thymidine	0.35

Once the desired level of hGH had been reached, continuous flow of fresh sterile medium was started, and product minus microcarriers retained by the stainless steel mesh removed at the same rate. With a cell count of 5×10^6 it was possible to maintain at dilution rate of .125 hr^{-1} with an hGH concentration of 60mg/l. The impellor shown was of the anchor type, obviously other configurations of impellor could be used providing the shear forces were low and did not create too much shear and lyse the cells. Shear generated is a function both of the design of impellor and rotational speed. In the configuration shown in Fig. 1, mass transfer of oxygen and CO_2 has to occur across the surface of the culture. Thus it is possible to accurately determine the interfacial area for mass transfer. Most mammalian cell lines have oxygen requirements of the order of 0.1-1.0 millimoles $O_2/10^6$ cells/hr. This means that if

surface aeration alone is relied on for oxygen mass transfer, at a cell density of 10^6 cells/ml, oxygen limitation is likely to occur if the ratio of surface area for transfer to liquid volume is less than 0.15 cm^{-1}.

In order to maximise product concentrations and productivities of recombinant proteins it is necessary to operate at as high a concentration of cells as possible. In order to avoid oxygen limitation at higher cell densities it may be necessary to bubble a gas phase, oxygen enriched if necessary, into the vessel. In this instance the gas is bubbled into the collection region of the reaction vessel. Another solution is to separate the production and mass transfer functions. This is shown in Fig. 2. Cells are grown in reactor 1. Medium from this reactor, filtered to remove cells, is pumped into the aeration chamber 13 where CO_2 is removed and oxygen transferred to the medium. Oxygenated medium is then pumped back to the production vessel. The rate of circulation of medium and relative volumes of the two reactors are functions of the cell concentration and the specific oxygen rates of the cells in reactor 1. It is possible to feed fresh sterile medium to the system and collect product on either a batch or continuous basis using the addition and removal point shown in Figure 2. For CHO cells at a density of 5 x 10^6 cells/ml it is necessary to circulate the medium at a rate which replaces the volume of reactor 1 to 7 times per hour. With a kla value in the second reactor of 40 hr^{-1}, the oxygenation reactor may be as small as 1/10 the value of the production reactor. By incorporating a second compartment inside reactor 1, consisting of a mesh which excludes the beads and cells, it is possible to oxygenate the medium within the compartment which is then mixed back with the bulk medium by the mixing action within the reactor providing oxygenated medium to cells throughout the

reactor. In this example cells attached to microcarriers have been described. Obviously the system could be operated where the cells had been immobilised by other methods, such as flocs, porous supports, micro encapsulation etc.

Example 2

This example makes use of the air lift principle, whereby a gas phase is used to promote low shear mixing as well as providing the gas phase for mass transfer. A gas phase is bubbled into the bottom of one section of the reactor, (the riser section), which is connected at the top of the reactor to the downcomer section. The introduction of the gas phase into the riser section lowers the density in this section, resulting in the de-aerated higher density culture in the downcomer displacing the lower density culture hence promoting gentle mixing in the reactor. It is possible to join the downcomer and riser sections in several reactor configurations. One configuration, having the riser as a concentric tube 14 inside a cylindrical reaction vessel 15 is shown in Fig. 3. This configuration has been used previously for the growth of hybridoma cells. It has been shown that it is possible to use the configuration for growth and product formation by CHO cells attached to microcarriers with gas rates as low as 0.02 VVM. The gas phase used is basically air, which can be supplemented using the usual control methods with carbon dioxide or ammonia, to maintain culture pH. If necessary, oxygen can also be added to the gas phase to maintain the desired level of dissolved oxygen without using excessively high gassing rates. At the low gassing rates used in this work, foaming problems are minimal; however if foaming occurs in the initial stages, e.g. when the cells are growing on serum containing medium, it is possible to control it with the metered addition (using a conductivity

sensor or timed addition) of a silicone based antifoam e.g. antifoam C, or other antifoams if they do not interfere with cell growth or product formation. Once the growth stage has occurred and the microcarrier beads are confluent, the cells are washed in 1 volume of PBS, using a stainless steel mesh to maintain the microcarriers. The reactor is then filled with essentially serum-free medium containing a copolymer of propylene oxide and ethylene oxide (Pluriol PE 6800) for the production of hGH. This medium consisted of the following:

APPENDIX 2

Appendix 2

INORGANIC SALTS	mg/l
$CaCl_2$ (anhyd.)	162.15
$CuSO_4 . 5H_2O$	0.0013
$Fe(NO_3)_3 . 9H_2O$	0.05
$FeSO_4 . 7H_2O$	0.417
KCl	352.5
KH_2PO_4	30.62
$MgCl_2$ (anhyd.)	23.33
$MgSO_4$ (anhyd.)	61.48
NaCl	6958.5
$NaHCO_3$	1338
$NaH_2PO_4 (H_2O)$	62.5
$ZnSO_4 . 7H_2O$	0.432
$Na_2HPO_4 . 7H_2O$	125
AMINO ACIDS	
L-Alanine	9.0
L-Asparagine.H_2O	15.0
L-Arginine.HCl	253.0
L-Aspartic acid	13.0
L-Cysteine HCl.H_2O	35.13
L-Cystine.2HCl	31.29
L-Glutamic acid	15.00
L-Glutamine	438.0

Glycine	23.0
L-histidine HCl.H_2O	42.0
L-Isoleucine	56.4
L-Leucine	65.6
L-Lysine HCl	109.5
L-Methionine	19.5
L-Phenylalanine	38.0
L-Proline	35.0
L-Serine	31.5
L-Threonine	59.4
L-Tryptophan	10.0
L-Tyrosine (disodium salt)	59.83
L-Valine	58.7
VITAMINS	
Ascorbic acid	7.5
Biotin	0.0037
D-Ca pantothenate	2.119
Choline chloride	8.98
Folic acid	2.66
i-Inositol	12.61
Nicotinamide	2.02
Pyridoxal HCl	2.03
Riboflavin	0.22
Thiamine HCl	2.169
Vitamin B_{12}	0.68
OTHER COMPONENTS	
D-Glucose	3151
HEPES	3570
Sodium pyruvate	110
Hypoxanthine (disodium salt)	2.7
Linoleic acid	0.045
Putrecine 2HCl	0.081
Thioctic acid (Lipoic)	0.103
Thymidine	0.35
Pluriol PE 6800	2000

With a cell concentration of 10^7 cells/ml, it was possible to run the reactor in a continuous fashion and produce a product stream at a dilution rate of 0.15 hr^{-1} and an hGH concentration of 80 mg/l. The reactor could also be run in the fed batch or repeated batch mode, where part or all of the contents could be removed from the reactor via the stainless steel mesh, and fresh media replaced either in a batch or continuous fashion. Using this technique, it was possible to obtain product stream with hGH concentrations up to 250 mg/litre.

In this example it is possible to bubble a gas directly in the culture medium as the medium contains the non-toxic, low-foaming, non-ionic compound Pluriol PE 6800. This compound is obtained by the copolymerization of propylene oxide and ethylene oxide. The presence of this compound in the culture medium enables aeration without causing the cells to disassociate from the support medium.

CLAIMS:

1. An essentially serum-free tissue culture medium characterized in that the culture medium includes in solution a non-toxic polymer which acts as a cell protective agent by reducing film drainage around the cells.

2. An essential serum-free tissue culture medium as claimed in claim 1 in which the polymer is either polyethylene glycol or polyvinyl pyrollidone.

3. An essential serum-free tissue culture medium as claimed in claim 1 in which the polymer is a polymer which contains one or more alkylene oxides.

4. An essentially serum-free tissue culture medium as claimed in claim 3 in which the alkylene oxides contain 2 or 3 carbon atoms.

5. An essentially serum-free tissue culture medium as claimed in claim 3 or claim 4 in which the polymer is a copolymer of propylene oxide and ethylene oxide.

6. An essentially serum-free tissue culture medium as claimed in any one of claims 3 to 5 in which the polymer of alkylene oxides is present in a concentration of 0.1 to 1.0%.

7. An essentially serum-free tissue culture medium as claimed in any one of claims 1 to 6 in which the tissue culture medium is essentially protein-free.

8. A method of culturing a mammalian cell which requires attachment comprising the following steps:

1. Culturing the mammalian cells in a serum containing medium in a reaction vessel provided with a support medium to which the cells will attach until the cells have attached thereto;
2. Decanting the serum containing medium;
3. Replacing the decanted medium with an essentially serum-free medium; and
4. Aerating the culture in a manner such that the

mammalian cells are not directly exposed to the aeration, or directly aerating the culture containing the mammalian cells wherein when the aeration is direct the essentially serum-free medium is as claimed in any one of claims 1 to 7.

9. A method as claimed in claim 8 in which the reaction vessel is divided into a culture region and a cell-free region, the culture region being separated from the cell-free region by means of a filter through which culture medium but not cells may pass.

10. A method as claimed in claim 8 or claim 9 in which the aeration of the culture is conducted by bubbling a gas into the cell-free region of the reaction vessel.

11. A method as claimed in claim 8 or claim 9 in which the aeration of the culture is achieved by aerating essentially serum-free medium free of cells in an aeration chamber remote from the reaction vessel, the aerated medium then being transferred into the reaction vessel.

12. A method as claimed in claim 11 in which the essentially serum-free medium free of cells which is subjected to aeration is, at least in part, medium taken from the cell-free region of the reaction tank.

13. A method as claimed in claim 8 or claim 9 in which the aeration is carried by means of a lift present within the reaction vessel.

14. A method as claimed in any one of claims 8 to 13 in which the mammalian cell is genetically engineered to contain DNA encoding a polypeptide, such that growth of the cells results in production of the polypeptide.

15. A method as claimed in claim 14 in which the polypeptide is recovered and purified from culture medium taken from the cell-free region of the reaction vessel.

16. A method as claimed in claim 14 or claim 15 in which the DNA coding for the polypeptide is under the control of an inducible promoter, the promoter being induced by a

component of the essentially serum-free medium.

17. A method as claimed in any one of claims 14 to 16 in which the mammalian cells are a continuous cell line.

18. A method as claimed in claim 17 in which the continuous cell line is Chinese hamster ovary cell line.

19. A method as claimed in any one of claims 14 to 18 in which the polypeptide is human growth hormone.

20. A method as claimed in any one of claims 8 to 19 in which the essentially serum-free medium is also essentially protein-free.

21. A method as claimed in any one of claims 8 to 20 in which the culture is agitated in a manner such that shear forces generated are low.

22. A method as claimed in any one of claims 14 to 21 in which the polypeptide is recovered from culture medium continuously removed from the reaction vessel, and fresh essentially serum-free medium is continuously added to the reaction vessel.

23. An essentially serum-free tissue culture medium substantially as hereinbefore described with reference to Example 2.

24. A method of culturing a mammalian cell which require attachment substantially as hereinbefore described with reference to Example 1 or 2.

AMENDED CLAIMS

[received by the International Bureau on 29 January 1988 (29.01.88);
original claims 1-24 replaced by new claims 1-22 (3 pages)]

1. A method of culturing mammalian cells which require attachment comprising the following steps:

1. Culturing the mammalian cells in a serum containing medium in a reaction vessel provided with a support medium to which the cells will attach until the cells have attached thereto and grown to the desired cell concentration;
2. Decanting the serum containing medium;
3. Replacing the decanted medium with an essentially serum-free medium which includes in solution a non-toxic polymer which is capable of acting as a cell protective agent by reducing film drainage around the cells and the support medium; and
4. Aerating the culture.

2. A method as claimed in claim 1 in which the non-toxic polymer present in the serum-free medium is selected from the group comprising polyethylene glycol and polyvinyl pyrollidone.

3. A method as claimed in claim 1 in which the non-toxic polymer present in the serum-free medium is a polymer which contains one or more alkylene oxides.

4. A method as claimed in claim 3 in which the alkylene oxide contains 2 or 3 carbon atoms.

5. A method as claimed in claim 3 or claim 4 in which the polymer is a copolymer of propylene oxide and ethylene oxide.

6. A method as claimed in any one of claims 3 to 5 in which the polymer of alkylene oxides is present in the medium in a concentration of 0.1 to 1.0% by weight.

7. A method as claimed in claim 1 in which the reaction vessel is divided into a culture region and a cell-free region, the culture region being separated from the cell-free region by means of a filter through which

culture medium but not cells may pass.

8. A method as claimed in Claim 7 in which the aeration of the culture is conducted by bubbling a gas into the cell-free region of the reaction vessel.

9. A method as claimed in Claim 8 in which the aeration of the culture is achieved by aerating essentially serum-free medium free of cells in an aeration chamber remote from the reaction vessel, the aerated medium then being transferred into the reaction vessel.

10. A method as claimed in claim 9 in which the essentially serum-free medium free of cells which is subjected to aeration is, at least in part, medium taken from the cell-free region of the reaction tank.

11. A method as claimed in any one of claims 1 to 7 in which the aeration is carried out by means of a gas lift present within the reaction vessel.

12. A method as claimed in any one of claims 1 to 11 in which the mammalian cell is genetically engineered to contain DNA encoding a polypeptide, such that growth of the cells results in production of the polypeptide.

13. A method as claimed in claim 12 in which the polypeptide is recovered and purified from culture medium taken from the cell-free region of the reaction vessel.

14. A method as claimed in claim 12 or claim 13 in which the DNA coding for the polypeptide is under the control of an inducible promoter, the promoter being induced by a component of the essentially serum-free medium.

15. A method as claimed in any one of claims 12 to 14 in which the mammalian cells are a continuous cell line.

16. A method as claimed in claim 15 in which the continuous cell line is Chinese hamster ovary cell line.

17. A method as claimed in any one of claims 12 to 16 in which the polypeptide is human growth hormone.

18. A method as claimed in any one of claims 1 to 17 in which the essentially serum-free medium is also

essentially protein-free.

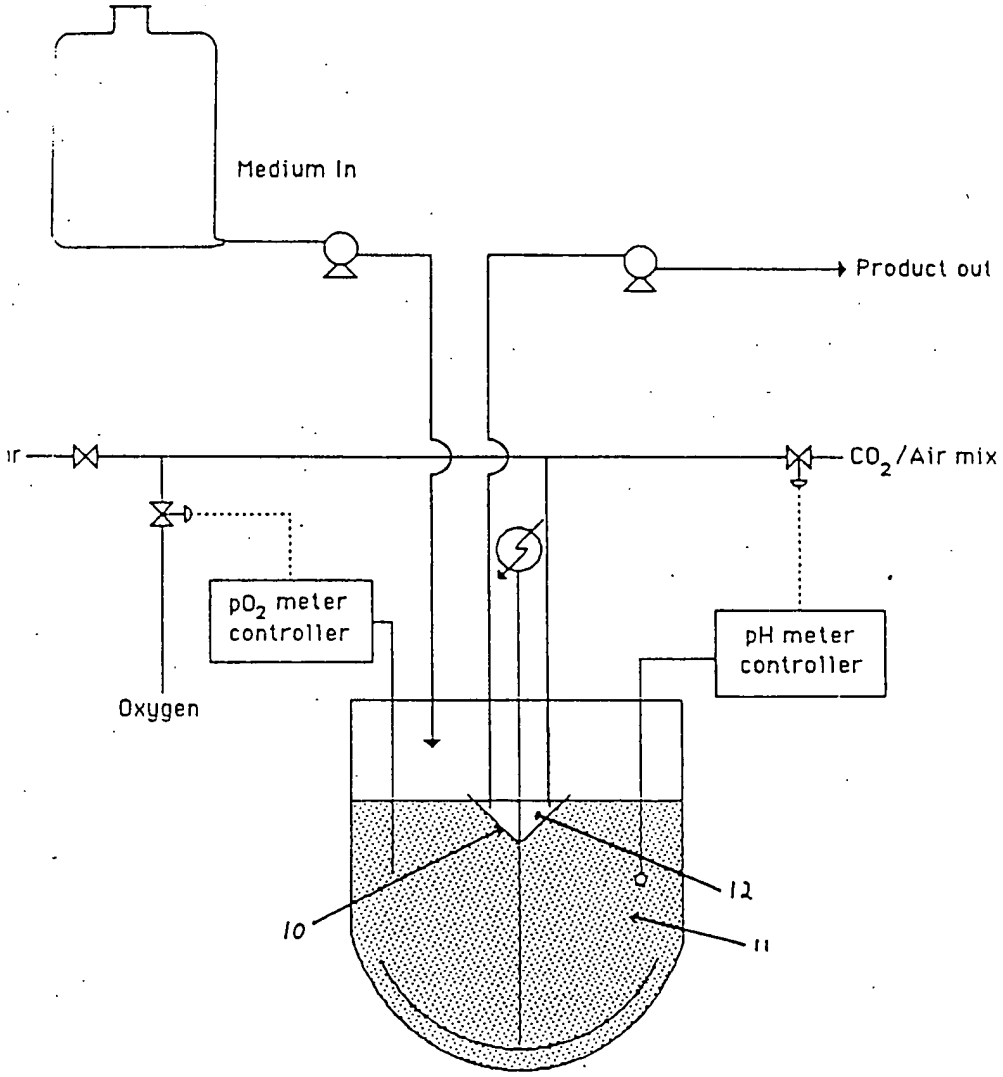
19. A method as claimed in any one of claims 1 to 18 in which the culture is agitated in a manner such that shear forces generated are low.

20. A method as claimed in any one of claims 12 to 19 in which the polypeptide is recovered from culture medium continuously removed from the reaction vessel, and fresh essentially serum-free medium is continuously added to the reaction vessel.

21. A method of culturing a mammalian cell which require attachment substantially as hereinbefore described with reference to Example 1 or 2.

22. A method of culturing mammalian cells which require attachment comprising the following steps:

1. culturing the mammalian cellsin a serum containing medium in a reaction vessel provided with a support medium to which the cells will attach until the cells have attached thereto and grown to the desired cell concentration;
2. decanting the serum containing medium;
3. replacing the decanted medium with an essentially serum-free medium; and
4. aerating the culture in a manner such that the mammalian cells are not directly exposed to the aeration.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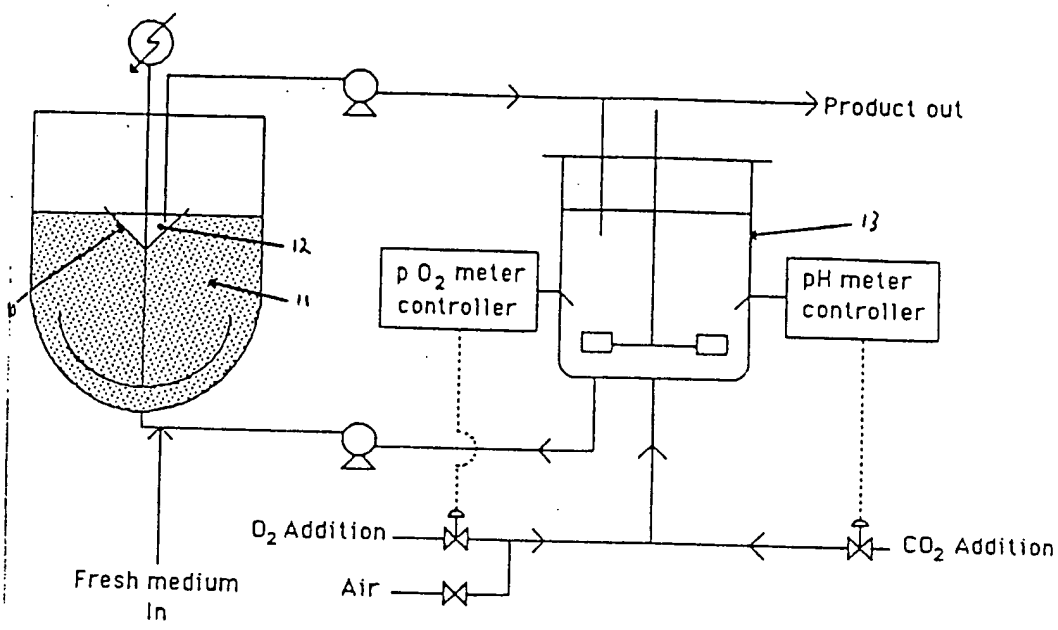
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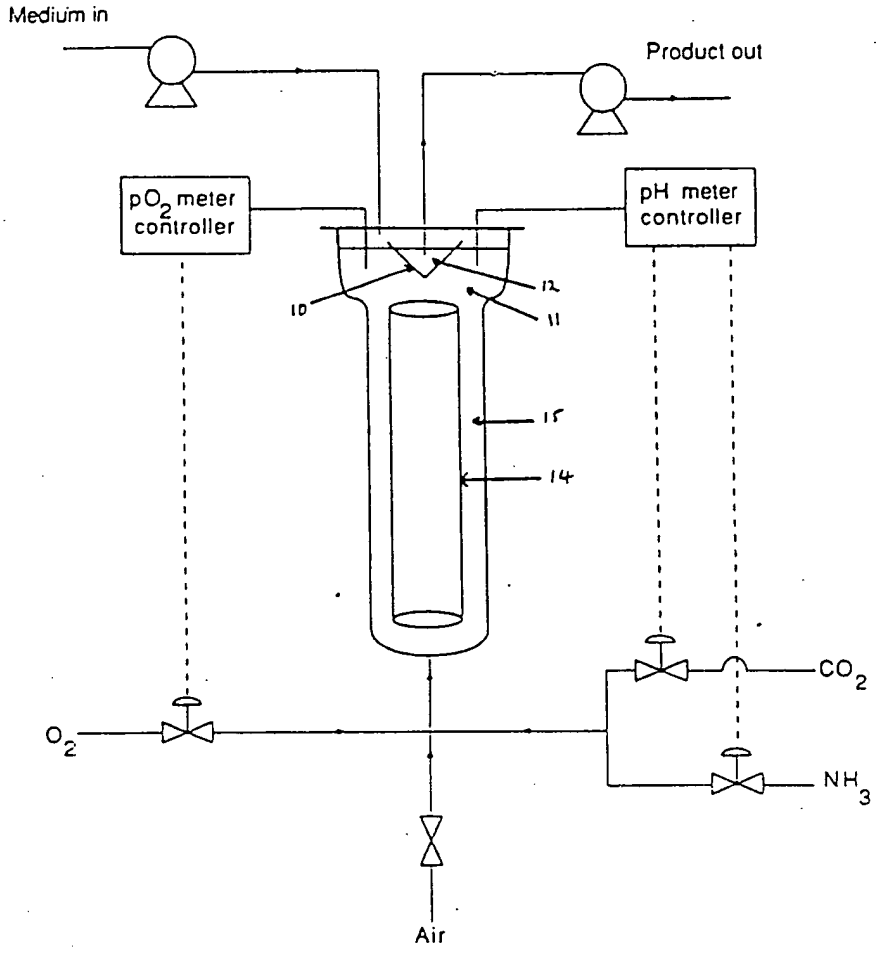
Reactor 2

Figure 3.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INTERNATIONAL SEARCH REPORT

International Application No PCT/AU 87/00248

I. CLASSIFICATION OF SUBJECT MATTER

According to International Patent Classification (IPC) or to both National Classification and IPC

Int. Cl. C12N 5/00, 5/02, 15/00

II. FIELDS SEARCHED

Minimum Documentation Searched

Classification System	Classification Symbols
IPC	C12N 5/00, 5/02
US Cl.	435/41

Documentation Searched other than Minimum Documentation to the Extent that such Documents are Included in the Fields Searched

AU : IPC as above

III. DOCUMENTS CONSIDERED TO BE RELEVANT

Category	Citation of Document, with indication, where appropriate, of the relevant passages	Relevant to Claim No.
X	AU,B, 54456/80 (542303) (NATIONAL RESEARCH DEVELOPMENT CORPORATION) 17 July 1980 (17.07.80) See page 6, lines 4-10	(1-2)
P,X	Derwent Abstract Accession no. 87-133257/19, Class B04, JP,A, 2074284 (TEIJIN KK) 6 April 1987 (06.04.87)	(1-2)
X,Y	Developments in Biological Standardization v 55, 1983, published 1984 by Karger (Basel) A. Mizrahi 'Oxygen in Human Lymphoblastoid Cell line Cultures and Effect of Polymers in Agitated and Aerated Cultures', pages 93-102, especially page 101	(1-24)
Y	US,A, 4390623 (FRABRICIUS et al) 28 June 1983 (28.06.83) See claim 1	(8)
A	AU,A, 38571/85 (DAMON BIOTECH, INC.) 22 August 1985 (22.08.85)	
A	AU,A, 22326/83 (BIO-RESPONSE, INC.) 21 June 1984 (21.06.84)	
A	AU,B, 75269/81 (546555) (CHLORELLA INDUSTRY CO. LTD) 22 April 1982 (22.04.82)	

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"T" later document published after the international filing date or priority date and not in conflict with the application but cited to understand the principle or theory underlying the invention

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"Y" document of particular relevance; the claimed invention cannot be considered to involve an inventive step when the document is combined with one or more other such documents, such combination being obvious to a person skilled in the art

"&" document member of the same patent family

IV CERTIFICATION

Date of the Actual Completion of the International Search	Date of Mailing of this International Search Report
17 November 1987 (17.11.87)	(30-11-87) 30 NOVEMBER 1987

International Searching Authority	Signature of Authorized Officer
Australian Patent Office	